KOREAN J. FOOD SCI. TECHNOL.
Vol. 21, No. 5, pp. 624~632 (1989)

Nonrandom Combination of Fatty Acid and Alcohol Moieties in Wax Esters from *Liza Carinata* Roe

Yong-Goe Joh, Kyeong-Hee Lee and Yeon-Joo Cho
Department of Food Science and Nutrition, Dong-A University, Pusan

Abstract

Lipids of *Liza carinata* roe were extracted and separated into detailed lipid classes by column chromatography. About 57-62% of the total lipids consisted of wax esters in which saturated and unsaturated fatty alcohols combined with fatty acids with up to six double bonds. Between the even-numbered wax ester peaks in gas-liquid chromatography, ones with odd chain lengths such as C31, C33 and C35 were eluted in appreciable amounts. Isomers composed of different fatty acids and alcohols at a given chain length were not resolved on 1.5% OV-17 column. The principal component of wax esters in sample A were C32, C34 and C30 (45.0%, 19.2% and 12.2%), followed by C36 and C38 length (9.5% and 4.7%), while those in sample B were mainly occupied by C34, C32 and C36 length (36.3%, 31.4% and 14.5%) with minor components C30 and C38 length (5.2% and 3.4%). The wax esters were not a random combination of constituent fatty acids and alcohols. With increase in boiling temperature the wax esters increased slightly in viscosity over the unboiled, showing a tendency toward randomness, and finally were completely randomized at 360 °C for 40 minutes. The enzymes involved in wax ester biosynthesis seemed to have high selectivity for chain length of fatty acids and alcohols.

Key words: nonrandom, randomizing, *Liza carinata*, even-numbered chain length, wax ester.

Introduction

Wax esters are harder to digest and absorb than any other lipids[1], and they often cause seborrhea symptoms[2] in humans when ingested in quantity.

However, since wax esters from marine animals generally exist in the liquid state, they are readily available to many industrial fields. Their most promising uses are for cosmetics, extreme-pressure and/or temperature lubricants, and leather tanning[3]. The major current sources of a liquid wax esters mixture are confined to the protected sperm whale[4,5] (*Physeter macrocephalus* and *P. catodon*) and the desert shrub jojoba[6] (*Simmondsia chinensis*[Link]Schneider). But these sources can not meet the great demand for liquid wax esters these days.

Corresponding author: Yong-Goe Joh, Department of Food Science and Nutrition, College of Natural Science, Dong-A University, 840 Hadan-dong, Saha-gu, Pusan 604-714

To solve this problem, more attention must be paid to the exploitation of new sources: deep sea fish such as the orange roughy[7] (*Hoplostethus atlanticus*), the black oreo[7] (*Allocyttus* sp.), the small spined oreo[7] (*Pseudocyttus maculatus*) and the *Laemonema longipes*.[8] Recently, many studies on the production of wax esters by microorganisms have been reported[9,10] on.

In a previous paper[11], we have indicated that *Liza carinata*, a species of the *Mugilidae* family, contains a high percentage of wax esters in its roe lipids and their fatty acids are found to be more highly unsaturated than those of the triglycerides in roe and muscle lipids.

It is of importance to clarify the physical and chemical properties of the wax esters from *L. carinata* roe lipids for more effective utilization in industrial fields.

The present study attempts to investigate the composition of molecular species and to compare by gas-liquid chromatography (GLC) the found composition of wax esters with a theoretically cal-

culated one, assuming a random combination of fatty acids and alcohols in the wax esters.

Experiment

Materials

Liza carinata bearing eggs were bought at Dadae-Po Fish Market, Pusan City, Korea, on May 17 of 1988. The fish A and B were 20.9 cm and 19.0 cm long, and weighed 113.9g and 93.6g, respectively. The fish roe A and B (sample A and B) from fish A and B weighed 44.3g and 36.8g, respectively.

All solvents were of extra pure grade (Oriental Chemical Industry Co., Ltd., Seoul, Korea) and were redistilled before use. 10% BF_3-methanol was obtained from Fluka A.G. (CH-9470 BUCHS, packed in Switzerland). Standard fatty acid methyl esters of C14:0, C15:0, C16:0, C16:1ω6, C16:2ω4, C17:0, C17:1ω8, C18:0, C18:1ω9, C18:2ω6, C18:3ω6, C18:3ω3, C19:0, C19:1ω9, C19:2ω6, C20:1ω9, C20:2ω6, C20:3ω6, C20:4ω6, C20:5ω3, C22:4ω3, C22:5ω3 and standard alcohols, C14:0, C15:0, C16:0, C16:1ω7, C17:0, C18:0, C18:1ω9, C19:0 and C20:0, were purchased from Nu Check Pre., Inc. (Elysian, Minnesota, U.S.A.). Standard wax esters of palmityl laurate (C28), palmityl myristate (C30), palmityl palmitate (C32), stearyl palmitate (C34), stearyl stearate (C36), stearyl arachidate (C38), behenyl stearate (C40) and behenyl arachidate (C42), were also obtained from Nu Check Pre., Inc. Capryl margarate (C29), lauryl margarate (C31), myristyl margarate (C33), stearyl margarate (C35), arachidyl margarate (C37), behenyl margarate (C39) and lignoceryl margarate (C41) were synthesized from their components at 300 °C for 10-20 hours under reduced pressure (10-15mm Hg) and purified by fractional distillation for GLC standards of odd-numbered wax esters[12].

Lipid extraction and classification

The roe samples were ground with sea sand, and total lipids were extracted with a mixture of chloroform-methanol (2:1, V/V) in a Waring Blendor following the procedure of Bligh and Dyer[13].

Total lipids were fractionated into subclasses by silicic acid column chromatography according to the method described in the previous papers[11,16].

Wax ester hydrolysis[11]

Wax esters purely obtained were saponified by refluxing in 1N ethanolic potassium hydroxide solution containing traces of BHA as an antioxidant in a hot water bath (85 °C) for 1 hour. The hydrolyzate was thoroughly extracted with diethyl ether and unsaponifiables were rinsed with water until neutral. The combined aqueous phase and washings were acidified by 1N HCl and extracted with diethyl-ether, and the extract was washed with water and concentrated.

Preparation of methyl esters and acetates[11]

Fatty acids were esterified with 10% BF_3-methanol, and fatty alcohols were acetylated with a mixture of pyridine-acetic anhydride (1:1, V/V). Purity of these derivatives was checked on thin-layer chromatography prior to GLC analyses.

Silver nitrate impregnated column chromatography[14,15]

For the purpose of identification and resolution of their unknown and tailing peaks on GLC, fatty acid methyl esters were further classified into subfractions on a silicic acid column containing 20% silver nitrate by solvents of hexane-benzene by increasing the ratio of benzene to hexane. The subfractions collected were analyzed on GLC before and after hydrogenation.

Hydrogenation

Portions of wax esters and fatty acid methyl esters, if required, were dissolved in hexane containing 5% palladium charcoal catalyst, and were hydrogenated at room temperature for 12 hours under atmospheric pressure.

Table 1. Lipid composition of *L. carinata* roe by column chromatography[a]

Eluent	Volume (ml)	Fraction	Yield	
			Sample A mg(%)	Sample B mg(%)
Hexane	100	HC[c]	0.6(0.1)	1.0(0.2)
2% E-H[b]	500	WE	339.6(56.6)	314.3(62.2)
7% E-H	400	TG	106.8(17.8)	73.0(14.4)
13% E-H	300	FFA, ST	3.0(0.5)	3.7(0.7)
Ether	300	ST	34.8(5.8)	20.0(4.0)
5% $CHCl_3$-MeOH	200			
MeOH	200	PL	115.2(19.2)	93.7(18.5)

a) 0.55-0.60g of roe lipids were mounted on silicic acid columns packed with 20g of silica gel 60.
b) E-H: diethyl ether in hexane
c) HC: hydrocarbon, WE: wax ester, TG: triglyceride, FFA: free fatty acid, ST: sterol, PL: phospholipid

GLC analysis

Fatty acid methyl esters and alcohol acetates were analyzed by GLC following the methods described in the previous paper[11]. GLC analysis of wax esters was carried out on a Shimadzu GC-6A equipped with a hydrogen flame ionization detector and with 1m × 3mm coiled stainless column packed with 1.5% OV-17 on Shimalite W (60-80 mesh) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The detector and injection port temperature was held at 350 °C and the column temperature was programmed from 220 °C to 330 °C at the rate of 3 °C/min. The flow rate of carrier gas (nitrogen) was 20 ml/min.

Peak area percentage in all the runs was calculated on a Shimadzu Chromatopac C-E 1B.

Random Interesterification

Random interesterification of wax esters was performed following the procedures of Miwa[6] and Wada[18].

Results and Discussion

Quantity and composition of lipids in roe

The quantities of lipids in sample A and B amounted to 16.0% and 16.2%, respectively. As shown in Table 1, lipid compositions of the total lipids of roe are specified by a high proportion of wax esters, 55.6% and 62.2% in sample A and B, succeeded by phospholipids (19.2% and 18.5%) and triglycerides (17.8% and 14.4%). The levels of wax esters are higher than those found in the roes of croaker, *Cynoscion nebulosus*[19] (40.0%), mackerel, *Scomber japonicus*[20] (31.0%), and some fish of the *Stromateidei* family[21] such as *Centrolophus* sp. (39.0%) and *Stromateus maculatus* (37.0%), but are much lower than those found in mullet roe[19,20] (75-90%), in the muscle of deep-sea teleost fish[7] such as orange roughly (95%) and black oreo (92%), and of the castor oil fish[22], *Ruvettus pretiosus* (91.5%).

Small amounts of sterols and hydrocarbons were also present. Wax esters were contaminated with traces of sterol esters, but the latter did not interfere with the analyses of the wax esters.

Fatty acid and alcohol composition of wax esters

The composition of fatty acids and alcohols from the roe wax esters is shown in Table 2. Values obtained from GLC analyses were averages from three optimized runs.

The fatty acid composition of wax esters is characterized by high level of monoenoic acids and polyenoic acids; C16:1 acid is the most abundant component (25.9% and 27.3%), followed by C18:1 acid (14.7% and 18.5%) and C20:5ω3 acid (16.3% and 9.6%) in sample A and B. The wax esters of *L. carinata* roe have a higher proportion of polyenoic acids than ever reported for marine wax esters[4,5,7] mainly used for industrial purposes.

It should be noted that the ratio of C16:1 to

Table 2. Composition of fatty acid and alcohol from wax esters in roe

	Sample A				Sample B			
	Acid		Alcohol		Acid		Alcohol	
	%	mol%	%	mol%	%	mol%	%	mol%
C14:0	2.7	3.2	6.7	7.5	2.5	3.0	5.2	5.9
1	0.5	0.6	0.1	0.1	0.5	0.6	0.3	0.3
C15:0	0.3	0.3	3.2	3.5	0.2	0.2	3.4	3.6
1	0.5	0.6	0.4	0.4	0.7	0.8	0.5	0.5
C16:0	2.9	3.1	57.3	57.9	2.6	2.8	53.4	54.4
1	25.9	27.9	8.4	8.5	27.3	29.3	7.4	7.6
2	5.9	6.4			4.1	4.4		
3	2.9	3.2			2.5	2.7		
C17:0	0.8	0.8	1.5	1.4	1.3	1.3	2.2	2.1
1			1.1	1.1	0.8	0.8	1.0	1.0
3	1.0	1.0			1.2	1.2		
4	1.4	1.5			1.6	1.7		
C18:0	0.7	0.7	9.3	8.6	1.0	1.0	11.7	10.8
1	14.7	14.3	8.9	8.3	18.5	18.0	12.7	11.8
2	2.2	2.2			4.7	4.6		
3ω6	1.3	1.3			1.2	1.2		
3ω3	1.3	1.3			1.5	1.5		
4ω3	4.2	4.2			4.1	4.0		
C19:0	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4
1			1.0	0.9				
2	1.1	1.0			1.2	1.1		
C20:0			0.8	0.7			0.2	0.2
1	0.5	0.4	1.1	0.9	0.1	0.1	1.6	1.4
2	0.3	0.2			0.3	0.3		
3ω6	0.2	0.2			0.3	0.3		
4ω6	1.4	1.3			1.2	1.1		
4ω3	2.5	2.3			2.3	2.1		
5ω3	16.3	14.9			9.6	8.7		
C21:0								
5ω?	1.0	0.9			1.2	1.0		
C22:0								
4ω3	0.2	0.1			0.2	0.2		
5ω6	0.4	0.3			0.3	0.2		
5ω3	3.8	3.2			3.2	2.7		
6ω3	3.0	2.5			3.4	2.8		
Saturated	7.5		79.0		7.9		76.5	
Monoenoic	42.1		21.0		47.9		23.5	
Polyenoic	50.4				44.1			
Odd-numbered	6.4		7.4		8.5		7.5	
Even-numbered	93.6		92.6		91.4		92.5	

C18:1 acid in roe wax esters of sample A and B (1.8 and 1.5) is much greater than the 0.0-0.56 of wax esters in roe[23,24], muscle[25-28], liver[8] and other organs[21] of marine animals.

On the other hand, the fatty alcohol components are not nearly so diverse as those of the fatty acid, and do not contain long carbon chain (C22 and C24) and polyunsaturated fatty alcohols; the

prevailing components are C16:0, C18:0, C15:0 and C14:0 alcohol for the saturated, and C16:1 and C18:1 alcohol for the monounsaturated fatty alcohols; C16:0 alcohol is the main component in the fatty alcohol moieties of wax esters, 57.3% and 53.4% in sample A and B, respectively, followed by C18:0 alcohol (9.3%), C18:1 alcohol (8.9%) and C16:1 alcohol (8.4%) in sample A, and followed by C18:1 alcohol (12.7%), C18:0 alcohol (11.7%) and C16:1 alcohol (7.4%) in sample B. Significant amounts of medium odd-numbered carbon chain fatty alcohol were also present.

Malins[29] showed that *in vivo* 1-^{14}C-palmitic acid was incorporated into free fatty alcohols as well as into fatty alcohol and acid moieties of wax esters in dog fish (*Squalus acanthias*) and wax ester was formed by direct reaction of fatty acid and alcohol without any involvement of activated intermediate. Sand[30] verified that ^{14}C-labelled C16:0, C18:1, C18:2 and C18:3 acid were easily bioconverted into the corresponding alcohols in gourmis (*Trichogaster cosby*), which contains a high percentage of wax esters in its roe.

The combination of exclusively saturated and monounsaturated alcohols with a great variety of polyunsaturated fatty acids strongly suggests that saturated and monounsaturated fatty acids are preferentially amenable to enzymatic reduction of their carboxyl group in *L. carinata*.

Wax ester composition and nonrandomness in its biosynthesis

The combination of a wide range of fatty acids with several fatty alcohols poses a problem of distribution pattern similar to that of fatty acid combination in glycerides. Since the unsaturated components in natural wax esters were not completely resolved from the saturated counterparts as shown in Fig. 1, the wax esters to be tested were hydrogenated and analyzed by GLC (Fig. 2-a). Between the even-numbered chain peaks occurred another series of small ones due to odd-numbered chain components, but isomers containing different fatty acids and alcohols at a given chain

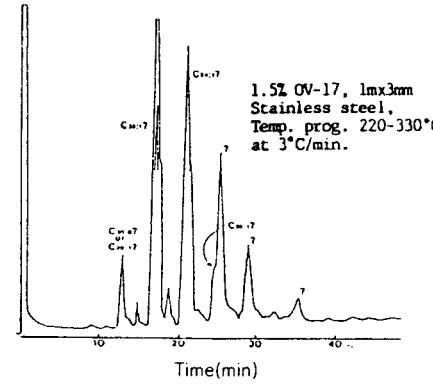

Fig. 1. Gas liquid chromatography of intact wax esters from *L. carinata* roe (Sample A).

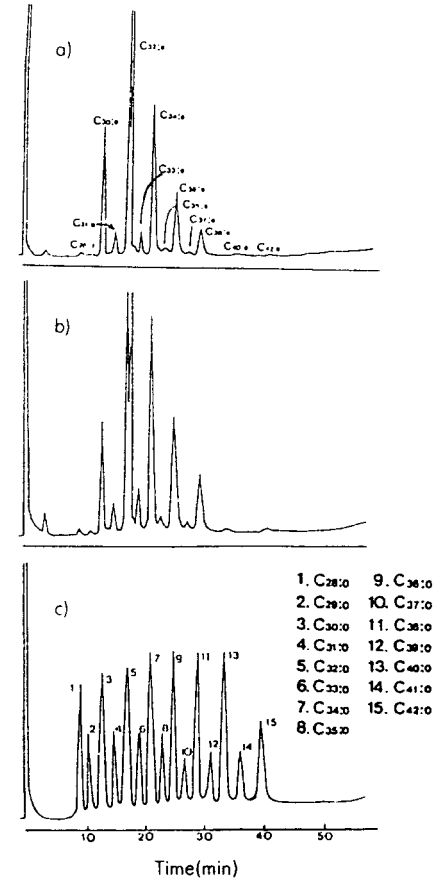

Fig. 2. Gas liquid chromatography of wax esters from *L. carinata* roe (Sample A) after hydrogenation.
Operation conditions of gas liquid chromatography are described in Experiment.
a): not randomized
b): randomized at 360°C for 40 min. before hydrogenation
c): standards

Table 3. Composition of wax esters by GLC and random combination of fatty acids and alcohols in wax esters

Carbon Number	Sample A (mol%)		Sample B (mol%)	
	found on GLC	calculation	found on GLC	calculation
C28	0.7	0.3	0.1	0.2
C29	0.2	0.2	tr.	0.2
C30	12.2	5.6	3.4	4.7
C31	3.0	2.5	1.4	2.7
C32	45.0	29.6	31.4	27.2
C33	2.9	4.4	4.3	5.9
C34	19.2	24.5	36.3	28.7
C35	0.8	3.2	2.2	3.7
C36	9.5	18.1	14.5	15.8
C37	0.8	1.8	0.9	1.8
C38	4.7	7.7	5.2	7.0
C39	0.3	0.5	0.2	0.5
C40	0.7	1.4	0.1	1.5
C41	tr.	0.1	tr.	tr.
C42	tr.	0.1	tr.	0.1
odd-numbered	8.0		9.0	
even-numbered	92.0		91.0	

tr: trace, below 0.1%

length were not resolved. This finding is strongly supported by Spencer[4] and Iyengar[19].

As listed in Table 3, the principal components in sample A are C32, C34 and C30 lengths (45.0%, 19.2% and 12.2%), followed by C36 and C38 lengths (9.5% and 4.7%), while those in sample B are represented with C34, C32 and C36 lengths (36.3%, 31.4% and 14.5%) with minor components C38 and C30 lengths (5.2% and 3.4%). These results contrast strongly with the longer chain lengths of C34-42 found in the oils of deep-sea fishes[7] such as orange roughly (*H. atlanticus*), black oreo (*Allocyttus* sp.), small spined oreo (*P. maculatus*) and another deep-sea teleost[8] (*L. longipes*), and also with those of C40-C44 found in jojoba[6] oil. Considerable amounts of odd-numbered chain components were also present in sample A and B (8.0% and 9.0%).

Challinor[31] and his co-workers reported that the contents of saturated, monoenoic and dienoic wax esters from sperm whale head oil were appreciably different from those calculated on the assumption of random combination. The discrepancy between the found and the calculated composition also has been observed in jaw fat of the Atlantic bottlenose dolphin[32], barley epicuticular wax[33] and jojoba seed oil[6].

On the other hand, wax ester compositions obtained from GLC analysis confirm with statistical combinations of fatty acids and alcohols in the muscle lipids of castor oil fish[22], *Ruvettus pretiosus*, lantern fish[26], *Lampanyctus ritteri*, in the liver oils of *L. longipes*[8], in the seed oils of sun flowers[34], and in the surface lipid of pea leaves[35].

Iyengar[19] indicated that in mullet roe the combination of chain lengths was random with a sample which contained about 10% each of C14 and C15 alcohol, but it was not random when each of these alcohols occurred at the level of 20%.

Though deviations between the former and the latter ones greatly fluctuate from sample to sample, theoretical values are not in agreement with the observed GLC results for sample A and B on the whole, when comparing only the total wax esters of a given chain length; the levels of C30 and C32 lengths in sample A and those of C32 and C34 in sample B are higher than expected from random combination of the fatty acid and alcohol constituents, whereas those of the components with longer chain lengths than C36 in both samples were less than expected. (Table 3).

This nonrandomness suggests that at a certain or all stage(s) during the development of *L. carinata* roe, the enzymes involved in wax ester biosynthesis have high selectivity for specific chain lengths of fatty acids and alcohols.

A change of the composition of wax esters toward randomness occurred by boiling; at 780 mm Hg under nitrogen, the wax esters were randomized in sand baths controlled at 250 °C, 300 °C and 360 °C, respectively. On cooling, the wax esters boiled at 250 °C did not show any change in physical and chemical characteristics compared with the controlled.

However, with further increase in boiling tem-

Table 4. Changes of molecular species composition of wax esters in sample A after random interesterification at 250°C, 300°C and 360°C for 20 minutes (mol%)

Carbon length	Before randomizing	Random interesterification					Calculated by combination
		250°C	300°C	360°C			
					a	b	
C28	0.7	0.4	0.4	0.5	0.4	0.3	0.3
C29	0.2	0.3	0.2	0.3	0.2	0.2	0.2
C30	12.2	12.7	8.7	6.2	6.0	5.8	5.6
C31	3.0	3.1	2.7	2.9	2.5	2.3	2.5
C32	45.0	44.9	41.6	37.5	30.8	30.9	29.6
C33	2.9	3.0	3.9	3.9	4.1	4.2	4.4
C34	19.2	18.9	21.4	23.9	24.8	24.8	24.5
C35	0.8	1.0	1.5	2.0	2.7	2.9	3.2
C36	9.5	9.3	11.9	12.6	17.6	17.4	18.1
C37	0.8	0.9	0.8	1.4	1.5	1.6	1.8
C38	4.7	4.5	5.9	6.6	7.2	7.5	7.7
C39	0.3	0.4	0.2	0.7	0.7	0.6	0.5
C40	0.7	0.6	0.8	1.5	1.5	1.5	1.4
C41	tr.	tr.	tr.	tr.	tr.	tr.	0.1
C42	tr.	tr.	tr.	tr.	tr.	tr.	0.1

a: for 40 minutes
b: for 100 minutes
tr.: trace, below 0.1%

perature the wax esters increased slightly in viscosity over the unboiled and showed a tendency toward randomness. In particular, when boiled at 360°C for 40 minutes, they were completely randomized as presented in Table 4 and Fig. 2-a, b and no further significant compositional variation of wax esters occurred with more prolonged boiling time (100 minutes) at 360°C though slight coloring could be observed.

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(Received Apr. 12, 1989)

등줄숭어 란유의 Nonrandom 분포를 한 왁스에스테르 조성에 관한 연구

조용계·이경희·조연주
동아대학교 식품영양학과

등줄숭어알 지질조성은 왁스에스테르(56.6-62.2%), 인지질(19.2-18.5%), 트리글리세리드(17.8-14.4%)의 순이었다. 왁스에스테르의 주요지방산은 C16 : 1(25.9-27.3%)>C18 : 1(14.7-18.5%)>C20 : 5ω3(16.3-9.6%)의 순이었으며, 알콜은 C16 : 0(57.3-53.3%)>C18 : 0(9.3-11.7%)>C18 : 1(8.9-12.7%)의 순이었다. 이중결합 2개 이상의 고도불포화 알콜은 전혀 검출되지 않았다. 홀수지방산(6.4-8.5%)과 알콜(7.4-7.5%)도 상당량 존재하였다. 수소첨가한 왁스에스테르는 탄소수별로 분리되었으나, 같은 탄소수를 가진 이성체는 분리되지 않았다. 왁스에스테르 분자종의 조성을 보면 시료 A의 경우는 C32(45.0%)>C34(19.2%)>C30(12.2%) 순이었고, 시료 B의 경우는 C34(36.3%)>C32(31.4%)>C36(18.1%)의 순이었다. 왁스에스테르는 구성지방산과 알콜이 nonrandom combination으로 에스테르화 되었으며, 이를 360℃에서 40분간 가열하면 완전히 random화 됨을 알 수 있었다. 등줄숭어에 존재하는 왁스에스테르의 생합성에 관여하는 효소는 기질인 지방산과 알콜 탄소수에 강한 특이성을 가지고 있음을 추측할 수 있었다.